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Marxen, Eva; Mosgaard, Mette Dalskov; Pedersen, Anne Marie Lynge; Jacobsen, Jette

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1 **Mucin dispersions as a model for the oromucosal mucus layer in *in vitro* and *ex vivo* buccal**
2 **permeability studies of small molecules**

3 *Eva Marxen^{a,1}, Mette Dalskov Mosgaard^{a,1,2}, Anne Marie Lynge Pedersen^b and Jette Jacobsen^a*

4 ^aDepartment of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen,
5 Universitetsparken 2, Copenhagen, Denmark, eva.marxen@sund.ku.dk, jette.jacobsen@sund.ku.dk

6 ^bDepartment of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen,
7 Nørre Allé 20, Copenhagen, Denmark, amlp@sund.ku.dk

8 ¹ These authors contributed equally to this work.

9 ²Present address: Department of Micro- and Nanotechnology, Technical University of Denmark,
10 Ørstedes Plads, Kgs. Lyngby, Denmark, medmo@nanotech.dtu.dk

11 Corresponding author:

12 Associate Prof. Jette Jacobsen

13 Department of Pharmacy, University of Copenhagen

14 Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

15 Email: jette.jacobsen@sund.ku.dk

16 Phone: +4535336299

17 **Abstract**

18 The mucus layer is believed to play a part in drug permeation across the oral mucosa. Human freeze-
19 dried saliva (HFDS) and porcine gastric mucin (PGM) was evaluated as model for mucus layer per
20 se or in conjunction with *in vitro* and *ex vivo* buccal permeability models.

21 Four small molecules (nicotine, mannitol, propranolol, caffeine) showed decreased permeability
22 across mucin dispersions, compared to controls, and a greater effect was seen with HFDS than with
23 PGM. Permeability of propranolol and caffeine across filter-grown TR146 cells was decreased by the
24 presence of mucin, whereas no effect was found on nicotine and mannitol. Incubation of porcine
25 buccal mucosa with mucin dispersions for 24 h compromised the integrity of the tissue, whereas 30
26 min incubation did not affect tissue integrity. Tissue incubation with mucin dispersions did not
27 decrease nicotine permeability. For the studied model drugs, it is concluded that mucin dispersions
28 constitute a minor barrier for drug diffusion compared to the epithelium.

29 **Keywords**

30 Mucus, permeability, barrier, buccal drug delivery, drug diffusion, absorption, small molecules

31

32 **Abbreviations**

33	BSA	Bovine serum albumin
34	HBSS	Hanks' balanced salt solution
35	HFDS	Human freeze-dried saliva
36	Log P	Logarithm of partition coefficient
37	Log D	Logarithm of distribution coefficient
38	MW	Molecular weight
39	P _{app}	Apparent permeability coefficient
40	PBS	Phosphate buffered saline
41	PGM	Porcine gastric mucin
42	SD	Standard deviation
43	TEER	Transepithelial electrical resistance

44 **1 Introduction**

45 Over the last years the interest in oromucosal drug delivery has increased due to advantages with this
46 administration route. The harsh environment of the gastrointestinal tract is avoided and hepatic first
47 pass metabolism is circumvented. Furthermore, the oral cavity is easily accessible for rapid self-
48 administration and the formulation can quickly be removed in case of adverse events [1]. The
49 epithelial surface of a mucosal membrane is covered by a mucus layer. The mucus layer has multiple
50 physiological functions such as lubrication, hydration, and tissue protection. Mucus is a complex
51 viscoelastic network, mainly consisting of water (95-99%) and mucins (1-5%) [2]. Mucins are
52 glycoproteins which may be susceptible to changes in salt concentration or temperature, which can
53 affect the mucin network and thereby the barrier properties of the mucus layer. It is believed that drug
54 permeation through the mucus layer is affected by interactions and entanglement with the mucin
55 network and by the unstirred water layer that mucus constitutes [3-5]. Mucus could retard drug
56 diffusion by interacting with the drug and by decreasing diffusion rate due to higher viscosity.
57 Reversely, mucus may increase solubility of the drug, and consequently increase the drug
58 concentration adjacent to the epithelium.

59 Only few oromucosal formulations are on the market, and this may be due to lack of standardized *in*
60 *vitro* methods to evaluate and optimize drug delivery systems [6]. Several models for assessing drug
61 permeability through oral mucosa are known, however, they lack the mucus layer. Permeability
62 across excised mucosal tissue can be studied in side-by-side diffusion cells such as modified Ussing
63 chambers or Franz cells [7]. To the authors' knowledge it has not been confirmed that the mucus layer
64 on the epithelium is intact after handling the excised tissue [8]. Filter grown cell cultures, such as
65 TR146 cells derived from human buccal carcinoma have also been used to model oral mucosa [9,
66 10]. However, these cells do not produce mucus. Cell cultures that produce a mucus layer, such as
67 HT29-MTX cells derived from human colon adenocarcinoma, form a single cell layer, and thus are

not suitable for modeling the oral mucosa, consisting of multiple cell layers [11]. Setups for studying drug transport across a mucus layer have previously been designed for Ussing chambers [12, 13]. However, they are either not suitable for liquid mucus or require several preparation steps for each replicate. Thus, there is a need for a simple setup containing mucus that can be used for high throughput studies.

Mucin can be obtained from mucus collected by gentle scraping of a mucosal membrane, from saliva, or from gastric fluid in animals or humans. Porcine gastric mucin (PGM) is commercially available as a crude mixture of mucin. Despite the gastric origin, PGM is commonly used to mimic mucus in the oral cavity [14, 15]. However, PGM may differ significantly from mucin found in the human saliva and on the oral mucosa, due to the difference in species and place of origin [16]. Furthermore, PGM has been through processing steps that may alter the properties of the molecules, thus there may be a need for a mucin source that is more similar to native oromucosal mucus.

Currently, oromucosal drug delivery is more feasible for small molecules, since they can diffuse the epithelial cell layers more easily than larger molecules. Therefore, four small molecules with different physicochemical properties were chosen as model drugs for this study; nicotine (MW = 162.2 g/mol; $\log D_{6.8} = 0.30$, calculated from $\log P = 1.43$ and $pK_a = 7.9$) [17], mannitol (MW = 182.2 g/mol; $\log P = -3.1$) [18], propranolol (MW = 259.3 g/mol; $\log D_{6.8} = 1.20$) [19], and caffeine (MW = 194.2 g/mol; $\log P = -0.07$) [20].

The aim of this study was to implement a mucin dispersion mimicking the mucus layer into *in vitro* and *ex vivo* permeability models and study the barrier properties of the mucin dispersion using small molecules. Thus, the permeability of nicotine, mannitol, propranolol and caffeine across a mucin dispersion, TR146 cells and porcine buccal mucosa was studied. The TR146 cells and the porcine buccal mucosa were incubated with mucin dispersions prior to the permeability experiments. Furthermore, the suitability of PGM and human freeze-dried saliva (HFDS) as sources of mucin was

evaluated. Implementation of a mucus layer will improve the predictability of the currently used permeability models, and this has to the authors' knowledge not previously been done.

2 Materials and methods

2.1 Materials

Potassium dihydrogen phosphate, calcium chloride, sodium hydrogen carbonate, sodium chloride and ortho-Phosphoric acid 85% were purchased from Merck KGaA (Darmstadt, Germany). Sodium phosphate monobasic anhydrous was obtained from Amresco (Solon, OH, USA). Potassium chloride was obtained from Riede-de Haën (Seelze, Germany). Hanks' balanced salt solution (HBSS) (10x), +CaCl₂, +MgCl₂ and 7.5% sodium bicarbonate was purchased from Gibco® life technologies (Grand Island, NY, USA). Nicotine bitartrate dihydrate was kindly donated from Fertin Pharma (Vejle, Denmark). Pearlitol® 160 (mannitol) was obtained from Roquette Pharma (Lestrem, France). Caffeine was purchased from VWR (Leuven, Belgium). [¹⁴C]-mannitol (57.1 mCi/mmol), [³H]-nicotine (80.4 Ci/mmol), [¹⁴C]-caffeine (54.9 mCi/mmol), [³H]-propranolol (18.6 Ci/mmol) and Ultima Gold™ liquid scintillation fluid were purchased from Perkin Elmer Inc. (Waltham, USA). Falcon 12-well tissue culture plates and cell culture inserts (polyethylene terephthalate membrane, 0.9cm² area, 0.4 mm pore size) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA). Bovine serum albumin (BSA), Mucin from porcine stomach, type II (PGM), (±)-propranolol hydrochloride, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Alcian blue 8GX, MTS-4-fluorescein, phenazine methosulfate and silicone oil were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Human freeze-dried saliva (HFDS) from pooled saliva samples (dialyzed and free of minerals) was kindly donated by the Department of Odontology, University of Copenhagen (Copenhagen, Denmark). The saliva was centrifuged at 2000 g for 10 min, dialyzed at 5°C for two days and finally lyophilized. Dialysis tubing visking, cellulose, type 36/32 inch, thickness 0.02 mm, width 44 mm, MWCO 14,000 was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Deionized water was

collected from Milli-Q water system, SG Ultra Clear 2002 from Evoqua Water Technologies LLC (Warrendale, PA, USA).

2.2. Methods

2.2.1 Preparation of mucin dispersions

A buffer, named saliva buffer, containing 5 mM KH_2PO_4 , 15 mM KCl, 1 mM CaCl_2 and 5 mM NaHCO_3 was prepared and adjusted to pH 6.8 [21]. PGM and HFDS were dispersed in saliva buffer and exposed to slow stirring at 5°C overnight. The concentrations of mucin varied in the experiments and are stated in the respective sections below.

2.2.2 Comparison of mucin sources

The mucin sources, PGM and HFDS, were visually compared in dry solid form. Furthermore, the pH of the two dispersions was measured. The viscosity of PGM and HFDS dispersions (2% w/v) were determined as described by [22]. Briefly, an AR-G2 plate and cone rheometer (TA instruments-Waters, New Castle, USA) was used with a 40 mm aluminum steel plate in diameter. A gap of 500 μm was selected (630 μL sample) and all the measurements were conducted at 37 °C. A protective casing, custom made at the Department of Pharmacy, University of Copenhagen (Denmark) was attached to the fixed heating plate and silicone oil (500 μL) was placed around the sample to prevent evaporation. The sample was equilibrated for 5 min before measurements were conducted. A steady state flow test to determine the viscosity was performed (shear rates 0.001-1000 s^{-1} , three consecutive measurements of 10 s with <5% variance). Four measurements were conducted per decade within a maximum time for each shear rate of 2 min (discarded if equilibrium was not reached within 2 min). TA Instruments Rheology Advantage Software (TA Instruments-Waters) was used to generate rheology data.

2.3 Permeability studies

139 2.3.1 Permeability across an isolated mucin dispersion

140 The new permeability device consisting of a test sample compartment in a tailor-made slider was
141 developed for the modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA, USA) to
142 enable drug permeation study through e.g. an isolated mucus layer.

143 The new permeability device consists of three parts as shown in Fig. 1; a cylinder placed between a
144 two piece slider. Dialysis membranes were rinsed in cold water and then soaked three times 5 min in
145 200 mL 100 °C hot water. The prepared dialysis membranes were placed between the slider and the
146 cylinder (positions shown with A in Fig. 1) and held in place by joining the parts. The mucus
147 dispersion was then added with a syringe through a small hole in the slider and cylinder. The diffusion
148 area was 0.50 cm² and the thickness of the mucin layer (cylinder length) was 6.0 mm. The
149 composition of the receptor and donor fluid is given in Table 1. The permeability of nicotine,
150 mannitol, propranolol and caffeine was studied across saliva buffer, 2% (w/v) PGM dispersion or 2%
151 (w/v) HFDS dispersion.

152 The study was conducted in modified Ussing chambers as previously described by Holm et al. [23].
153 Briefly, 2.0 mL of donor and receptor fluid was added to the respective compartments. Stirring was
154 ensured by supplying hydrated atmospheric air, and the temperature was kept at 36±1°C. Receptor
155 samples of 100 µL were taken from the receptor compartment at 5, 10, 20 and 30 min and then every
156 30 min up to 5 h. From the donor compartment, 100 µL was taken in triplicates at the start and end
157 of the experiment. After sampling the compartments were replenished. At the end of the experiment
158 a 100 µL sample was taken from the mucin dispersions or saliva buffer. Drug content was determined
159 in the dialysis membranes by rinsing off excess drug and placing the dialysis membrane in a
160 scintillation vial for measurement.

161

162 2.3.2 Permeability across TR146 cells

163 The TR146 cell line was provided by Imperial Cancer Research Technology (London, UK) and
164 cultivated and grown on filters as previously described [24]. On the first day of the experiment filter-
165 grown cells aged 25-27 days were washed on the apical side and the growth medium was changed on
166 the basolateral side. The cells were incubated on the apical side with 200 μ L 4% (w/v) PGM or HFDS
167 dispersion. Cells incubated with saliva buffer were used as a control. The cells were incubated at
168 37°C in 5% CO₂/95% air at 98% humidity for 24 h.

169 On the second day of experiment, the initial transepithelial electrical resistance (TEER) was measured
170 on the control cells (Endohm and voltmeter EVOM from World Precision Instruments (Sarasota, FL,
171 USA)). The composition of donor and receptor fluids is shown in Table 2. 1600 μ L receptor medium
172 was added to the basolateral side and 220 μ L donor solution was added to 200 μ L mucin dispersion
173 or saliva buffer on the apical side. The experiment was conducted at 37°C using a thermostatic
174 horizontal shaker, 100 rpm, (Edmund Bühler, swip Type KL-2) (Hechingen, Germany). 100 μ L
175 samples were taken from the basolateral side at time 10 min, 30 min, 45 min, 60 min and then every
176 30 min up to 240 min, and the compartment was replenished with receptor fluid. At 10 min a donor
177 sample of 20 μ L and at 240 min three donor samples of 20 μ L were taken. The first samples were
178 taken at 10 min to allow the donor fluid to mix with the mucin dispersion. After the permeability
179 experiment the cells were washed twice with HBSS buffer on both apical and basolateral side, TEER
180 was measured and a MTS-PMS viability test was conducted as described by Eirheim et al. [25].

181 2.3.2.1 Mucin attachment to cell surfaces

182 After 24 h incubation the mucin dispersions or saliva buffer was gently removed and the cells were
183 washed in 0.1 M phosphate buffered saline (PBS) pH 6.8. 200 μ L Alcian blue solution (1% (v/v)
184 Alcian blue and 3% (v/v) acetic acid in water) was added to the apical side and the setup was shaken
185 (100 rpm) for 5 min. The Alcian blue solution was gently removed and the cells were washed twice
186 in PBS pH 6.8. The cells were then examined under an Olympus BH2 light microscope (Olympus,

187 Tokyo, Japan) and representative pictures were taken with an AxioCam ERc5s (Zeiss, Jena,
188 Germany).

189 2.3.3 Permeability across porcine buccal mucosa

190 Porcine buccal mucosa was obtained from healthy experimental control pigs (approx. 30 kg Danish
191 Landrace/Yorkshire x Durox (D-LY)). Immediately after euthanization of the pigs the cheeks were
192 excised using a scalpel and placed in ice cold PBS pH 7.4. Within 3 h the excised cheeks were frozen
193 in 40% (w/v) glycerol and 20% (w/v) sucrose in PBS pH 7.4, and on the day of experiment the tissue
194 was thawed as described by Marxen et al. [26]. The buccal mucosa was trimmed with surgical scissors
195 and sliced to a thickness of $792\ \mu\text{m} \pm 88\ \mu\text{m}$ (n=36) using a Stadie-Riggs tissue slicer (Thomas
196 Scientific, Swedesboro, NJ, USA). The buccal mucosa was mounted on slider P2405 from
197 physiologic instruments Inc. (San Diego, CA, USA) (exposed area $0.40\ \text{cm}^2$). The sliders were placed
198 in the upper compartment of a desiccator, the epithelium facing upwards. The lower compartment of
199 the desiccator was filled with NaCl saturated water to ensure high humidity. 100 μL saliva buffer,
200 5% (w/v) PGM or HFDS dispersion was added to the apical surface of the tissue and incubated at 37
201 $^{\circ}\text{C}$ for 24 h, 30 min or the tissue was used immediately. When incubating for 24 h, the sliders were
202 placed in PBS pH 7.4 to keep the basolateral side of the tissue moist.

203 The compositions of the donor and receptor solutions are presented in Table 1. The permeability
204 experiment was conducted as described in Section 2.3.1, with few changes: After the experiment, the
205 tissue was dissolved in approximately 1.0 mL concentrated phosphoric acid, heated to 70°C and a
206 100 μL sample was taken for quantification of the radiolabeled model drugs.

207

208

209 2.4 Quantitative analysis

210 2 mL Ultima Gold™ liquid scintillation fluid was added to all samples before whirl-mixing.
 211 Quantitative analysis of [³H]-nicotine, [¹⁴C]-mannitol, [³H]-propranolol and [¹⁴C]-caffeine was
 212 performed by liquid scintigraphy using a Tri-Carb 2910TR Liquid Scintillation Analyzer (Perkin
 213 Elmer, Waltham, MA, USA).

214 **2.5 Data analysis**

215 Accumulated amount (Q, mol) of nicotine, mannitol, propranolol and caffeine appearing in the
 216 receptor compartment was plotted as a function of time (t). Steady state flux (J_{ss}, mol s⁻¹ cm⁻²) was
 217 calculated as the slope of the linear section of this curve (R² above 0.99 for isolated mucin layer and
 218 porcine buccal mucosa; R² above 0.95 for TR146 cells), using Equation 1, where A (cm²) is the area
 219 of diffusion.

$$220 \quad J_{ss} = \frac{dQ}{dt} * \frac{1}{A} \quad \text{Equation 1}$$

221 Steady state flux was obtained at different time intervals: Isolated mucin layer (90-210 min); porcine
 222 buccal mucosa (90-300 min); TR146 cells (45-150 min for nicotine and caffeine, 120-240 for
 223 propranolol and mannitol).

224 The apparent permeability coefficient (P_{app}) was calculated from Fick's first law of diffusion (J_{ss} =
 225 P_{app}·ΔC). When sink conditions are upheld, Fick's first law can be simplified to Equation 2, under the
 226 assumption that C_{donor} >> C_{receptor}. Thus P_{app} (cm s⁻¹) was calculated from Equation 2, where C_{0,donor}
 227 (mol cm⁻³) is the initial donor concentration.

$$228 \quad P_{app} = \frac{J_{ss}}{C_{0,donor}} \quad \text{Equation 2}$$

229 The total recovery (% of initial drug added) of the four model drugs was the sum of the accumulated
 230 drug amount (Q) at the end of the experiment in receptor compartment, donor compartment and drug
 231 amount in isolated mucin dispersion, saliva buffer, or porcine buccal mucosa, respectively. In the

cell studies the filter grown TR146 cells were used for viability testing, and thus the drug retained in cells was not quantified. Due to low recovery of propranolol, an additional experiment was performed to quantify the amount of propranolol in filter and cells.

2.6 Statistical analysis

Data in this study are presented as means with standard deviations (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to determine statistically significant difference between three or more means. An unpaired *t*-test was used to compare two means. Both tests were performed assuming equal variance and normal distribution of data. GraphPad Prism 7 for Windows, from GraphPad Software Inc. (La Jolla, CA, USA) was used for all statistical calculations. *P*-values below 0.05 were considered statistically significant.

3 Results and discussion

3.1 Comparison of mucin sources

The mucin sources appeared very different in solid dried forms. PGM is a fine brownish powder whereas HFDS has a bulky white fibrous structure. The pH of PGM dispersion after stirring overnight was 4.4 ± 0.3 ($n=9$) and the pH of HFDS dispersion was 6.6 ± 0.4 ($n=9$). A *t*-test showed that the pH of PGM dispersion was significantly different from pH of the HFDS dispersion, which was expected since PGM is gastric mucin, and thus originates from an acidic environment, whereas HFDS is derived from saliva with an average pH of 6.8. In the present studies the mucin dispersions were adjusted to pH 6.8 to mimic pH in the oral cavity. Mucin contains acidic functional groups, including sialic acid [27]. Adjusting pH will affect the degree of ionization of the acidic groups, thus a change in pH will likely affect the interactions between mucin strands, causing enlargement or compaction of the mucin network. Therefore, it is advantageous to use a mucin-type with an innate pH as close

255 to pH 6.8 as possible, thus HFDS would be a more suitable source for mucin used to mimic mucus in
256 the oral cavity.

257 The viscosity was determined to compare the shear thinning properties and viscosities of the two
258 types of mucin dispersion.

259 “The viscosity of a mucin dispersion demonstrates the degree of entanglement of mucin, and it is
260 known that increased viscosity correlates with decreased permeability through the dispersion [28].
261 The viscosities as a function of shear rate was determined for a 2% (w/v) PGM dispersion and a 2%
262 (w/v) HFDS dispersion. The results are presented in Fig. 2. The HFDS dispersion had a higher
263 viscosity than the PGM dispersion throughout the whole shear rate range (Fig. 2). Furthermore, a
264 decrease in viscosity with increasing shear rates (shear thinning) was observed for the HFDS
265 dispersion whereas the PGM dispersion had a constant viscosity at shear rates above 1 s^{-1} . At shear
266 rates below 1 s^{-1} several of the measurements on PGM gave negative values and were excluded. It
267 has been estimated that the movements in the oral cavity during speaking and swallowing correspond
268 to shear rates of approximately $1\text{-}160 \text{ s}^{-1}$ [29, 30]. The rheology measurements of the PGM dispersion
269 was considered acceptable, since the measurements above 1 s^{-1} shear rate was consistent, and this is
270 the range of shear rate of interest in oromucosal drug delivery. It is known that saliva and mucus
271 exhibit shear thinning properties [29], thus, based on the rheology results presented in Fig. 2 showing
272 HFDS to exhibit shear thinning unlike PGM, it appeared that HFDS was more similar to native mucus
273 and saliva. The observed differences may be due to differences between mucin from porcine gastric
274 fluid and human oral mucin [16]. Before the rheology measurements, pH was adjusted to 6.8, which
275 may affect protein conformation and interactions [31]. PGM originates from a gastric environment
276 with pH 1-2, and thus may be more affected by the pH increase compared to HFDS. Furthermore, it
277 is unknown what processing steps PGM has been subjected to, and the processing may have affected
278 the mucin molecules in a manner that decreased the gel forming properties. Measures of pH and

viscosity indicate that the HFDS is a more suitable mucin source than PGM, in terms of mimicking saliva and the mucus layer in the oral cavity. However, PGM, but not HFDS, is commercially available.”

3.2 Permeability studies

3.2.1 Permeability across an isolated mucin dispersion

The P_{app} was determined for the four model drugs permeating across the 2% (w/v) PGM, 2% (w/v) HFDS dispersions and the saliva buffer. The results are presented in Fig. 3, and the P_{app} values are given in Table 3. The P_{app} of both nicotine and mannitol across the saliva buffer was significantly higher than the P_{app} across the PGM dispersion, which in turn was higher than P_{app} across the HFDS dispersion (Fig. 3a and b). The P_{app} of propranolol across the saliva buffer was significantly higher than the P_{app} across the PGM dispersion and the HFDS dispersion. However, no significant difference was observed between the PGM and the HFDS dispersions (Fig. 3c). For caffeine the P_{app} across the saliva buffer and the PGM dispersion was significantly higher than the P_{app} across the HFDS dispersion. No significant difference was observed between the PGM dispersion and the saliva buffer (Fig. 3d).

Overall, it appears that the mucin dispersions act as a barrier to drug diffusion, and that HFDS constitutes a larger barrier than PGM. The diffusion of a drug is dependent on the molecular size of the drug and the viscosity of the medium. As described in Section 3.1 the viscosity of HFDS was higher, especially at low shear rates. The mucin dispersions are unstirred in this setup, thus the difference in viscosity may be an explanation for the decreased permeability found across HFDS. However, the mucin dispersions are not simply an unstirred water layer as they contain glycoproteins that potentially interact with the drug molecule. Several studies have shown the ability of mucin to

302 interact with a broad range of molecules [32, 33]. A detailed study of interactions between the model
303 drugs and mucin is beyond the scope of this study.

304 The cylinder containing the mucus dispersions or the saliva buffer was 6.0 mm thick. The thickness
305 of the mucus layer in the oral cavity shows regional variations, and has been determined to be
306 approximately 50 μm in the buccal area [34]. That is approximately 100 times thinner than in this
307 setup and it is likely that the permeability differences found will be negligible compared to the barrier
308 exerted by the epithelium.

309 3.2.2 Permeability across TR146 cells

310 The TR146 cells were incubated with 4% (w/v) of PGM, 4% (w/v) HFDS dispersion or saliva buffer
311 for 24 h, before determining P_{app} of the four model drugs across the cells.

312 The results are presented in Fig. 4, and the exact values are given in Table 3. No significant difference
313 was seen between the P_{app} of nicotine across the cells incubated with saliva buffer, PGM dispersion
314 and HFDS dispersion (Fig. 4a). The P_{app} of mannitol across the PGM dispersion was significantly
315 lower than P_{app} across the cells incubated with saliva buffer or the HFDS dispersion. However, no
316 significant difference was seen between P_{app} of mannitol across the saliva buffer and HFDS dispersion
317 (Fig. 4b). The P_{app} of propranolol and caffeine across the PGM and HFDS dispersion was significantly
318 lower than the P_{app} across saliva buffer. However, no significant difference was observed between the
319 PGM and HFDS dispersions (Fig. 4c and d).

320 The effects from the mucin dispersion on nicotine permeability across an isolated layer (Section
321 3.2.1), is not confirmed when the TR146 cells are present, indicating that the cell layers constitute a
322 larger barrier to nicotine, than the one provided by the mucin dispersions. Also the differences found
323 between HFDS and PGM dispersions, for propranolol and caffeine disappeared, which indicates that
324 the effect shown with a 6.0 mm mucin dispersion layer is larger than the barrier exerted by the mucus
325 layer *in vivo*. Moreover, the findings indicate that certain interactions between the drug molecules

and mucin did take place, as nicotine appeared less affected by mucin dispersions than the other model drugs. P_{app} for propranolol was decreased by 47% and 58% in the presence of PGM and HFDS dispersions, respectively, suggesting a higher degree of interaction with propranolol compared to the other model drugs. In accordance with previous findings we found that mucus constituted an increasing barrier to drug diffusion with increasing lipophilicity of the diffusing drug [11, 35]. This substantiates the need of a model as the one presented here, to determine whether the mucins affect the permeability of a drug molecule of interest.

3.2.2.1 Integrity and viability of TR146 cells

Following the permeability study TEER and MTS/PMS tests were used to determine the integrity and viability, respectively, of the TR146 cell layers. TEER values are an indication of electron transport particularly via the paracellular pathway. The initial TEER value of the TR146 cells was determined to be $145 \pm 43 \Omega \text{ cm}^2$ (n=19) after 24 h incubation with saliva buffer. After the 4 h permeability study, the TEER value for the TR146 cells incubated with PGM dispersion was $339 \pm 100 \Omega \text{ cm}^2$ (n=13), with HFDS dispersion was $196 \pm 31 \Omega \text{ cm}^2$ (n=11) and with saliva buffer was $157 \pm 59 \Omega \text{ cm}^2$ (n=14). The TEER value for PGM incubated cells were significantly higher ($p < 0.05$) than the initial TEER value, and no significant differences were found between HFDS and buffer incubated cells and the initial TEER value. The initial TEER value of $145 \Omega \text{ cm}^2$ indicate that the integrity of the TR146 cells was maintained after 24 h of incubation with saliva buffer, which is supported by Sander et al. who reported an initial TEER value of $151 \pm 38 \Omega \text{ cm}^2$ (n=119) on filter-grown TR146 cells. Furthermore, the integrity of the cell layers is not compromised during the permeability study. The TEER values were increased for the cells incubated with PGM indicating that incubation with mucin dispersions decreased electron transport. The findings are in line with Pontier et al. who showed that TEER values were increased after the HT29-MTX cells had intrinsically produced a mucus layer from mucus secreting goblet cells [36].

350 The integrity of the TR146 cell layers was supported by the relatively low P_{app} of mannitol. Mannitol
351 is routinely used as a marker for integrity of the cell layers, in particular the paracellular pathway.
352 Jacobsen et al. showed that mannitol permeability across TR146 cell layers grown for 30 days was
353 $5.2 \cdot 10^{-6}$ cm/s, and since the mannitol permeability found in this study was between 1.4 and $3.9 \cdot 10^{-6}$
354 cm/s (Table 3), the integrity of the cell layers seems to be maintained [24].

355 The MTS/PMS assay measures dehydrogenase activity in cells as a measure of cell viability. The
356 dehydrogenase activity after a 4 h permeability study in TR146 cells incubated with saliva buffer was
357 assumed to be 100% viable and the cellular viability of the TR146 cells incubated with PGM and
358 HFDS dispersions were determined relative to the cells incubated with saliva buffer. According to
359 Nielsen and Rassing [37], TR146 cells were not sensitive towards pH changes in the range 5.5-9.0,
360 or to osmolality changes in the range approximately 100-400 mOsm, thus it was assumed that the
361 saliva buffer would not affect viability of the TR146 cells. After 24 h incubation with mucin
362 dispersion and a 4 h permeability study the viability in cells exposed to nicotine and mannitol was
363 $106\% \pm 4\%$ (PGM) and $102\% \pm 2\%$ (HFDS), and in cells exposed to propranolol and caffeine the
364 viability was $107\% \pm 2\%$ (PGM) and $114\% \pm 21\%$ (HFDS).

365 MTS/PMS assays are often used to measure cellular toxicity of drugs. However, in this study it was
366 mainly used as a measure of the impact of incubation with PGM and HFDS dispersions compared to
367 saliva buffer (control). The selected concentrations of the model drugs have been validated in
368 previous studies, thus not expected to be toxic for the TR146 cells [10, 17, 38]. In this study the
369 viability of the cells did not decrease after exposure to the mucin dispersions, compared to incubation
370 with saliva buffer. The high integrity and viability supports the feasibility of the *in vitro* TR146 cell
371 model to study the effect of mucin on drug permeation.

372 3.2.2.2 Mucin attachment to cell surfaces

373 After incubation with mucin dispersions the attachment of mucin to the TR146 cells was qualitatively
374 examined by staining the cells with Alcian blue dye. Representative images of the stained cells are
375 presented in Fig. 5. Alcian blue stains the negatively charged groups in mucin at physiological pH
376 [39].

377 The TR146 cells incubated with saliva buffer (Fig. 5, left) showed scattered blue spots on the cell
378 layer surface. The staining of the TR146 cells incubated with PGM dispersion resembles the cells
379 incubated with saliva buffer solution. However, a few more densely stained areas were found on the
380 cells (Fig. 5, middle). The TR146 cells incubated with HFDS dispersion displayed larger stained areas
381 (Fig. 5, right). The Alcian blue stained cell surfaces clearly indicated a larger amount of mucin
382 attached to the cell surface when incubating with the HFDS dispersion. The attachment could be due
383 to interactions between the cell surface and mucin. The small amount of blue stains present on the
384 TR146 cells incubated with saliva buffer could indicate lack of washing during the staining procedure
385 or that other glycoproteins attached to the epithelial cell surface interacts with Alcian blue [40].

386 3.2.3 Permeability across porcine buccal mucosa

387 It was attempted to reintroduce a mucus layer to excised porcine buccal mucosa by incubating the
388 tissue with 5% (w/v) mucin dispersions. Due to limitations in tissue supply only nicotine and mannitol
389 were studied.

390 Initially, the tissue was incubated with saliva buffer or PGM for 24 h to allow time for interactions
391 between cell-bound mucin and mucin from the dispersions. The studies of mannitol (Fig. 6, b)
392 indicated that the integrity of the porcine buccal mucosa was compromised after 24 h incubation
393 compared to non-incubated tissue, for which mannitol amount was below the quantification limit (10x
394 noise/background). The lost integrity after 24 h incubation could be ascribed to hydration of the tissue,
395 which was further implied by visual swelling of the tissue after 24 h incubation and the permeability
396 study [41].

397 Secondly, porcine buccal mucosa was incubated for only 30 min with mucin dispersions or saliva
398 buffer. The choice of 30 min was based on previous studies allowing buccal mucosa to equilibrate
399 for 30 min, prior to the experiment [42]. As expected, after 30 min incubation with PGM, HFDS or
400 saliva buffer the permeated amount of mannitol was below the quantification limit (Fig. 6b),
401 indicating that tissue integrity was not compromised. Accordingly, the effect of mucin dispersions on
402 mannitol permeability across buccal mucosa cannot be determined.

403 The P_{app} values of nicotine after different incubation times were not affected by the possible loss of
404 tissue integrity, hence the P_{app} values of nicotine after 24 h incubation was not significantly higher
405 than P_{app} for non-incubated tissue (Fig. 6a). The P_{app} of nicotine after incubation for 30 min in PGM
406 dispersion was significantly higher than the P_{app} found with no incubation, 24 h in both saliva buffer
407 and PGM dispersion and after 30 min in HFDS dispersion. No significant difference was observed
408 between any of the other treatments. The significantly higher P_{app} value after incubation for 30 min
409 in PGM dispersion could indicate an enhancing effect on nicotine permeability. However, since none
410 of the findings from Section 3.2.1 and 3.2.2 indicate an enhancing effect of the PGM dispersion this
411 is believed to be coincidental.

412 Nicotine is more lipophilic than mannitol and permeates through both the transcellular pathway and
413 the paracellular pathway [43]. At pH 6.8, 93% of the nicotine will be mono-protonated. However, the
414 non-ionized form passing via the transcellular pathway contributes more to the apparent permeability
415 of nicotine than the mono-protonated form following the paracellular pathway [17, 43]. As a result,
416 the possible decrease in barrier integrity for mannitol has insignificant effect on the permeation of
417 nicotine, as it predominantly follows the transcellular pathway.

418 In Section 3.2.1 and Fig. 3 it is shown that PGM and HFDS decreases drug permeation across an
419 isolated mucin dispersion. This finding was not supported from the results with porcine buccal
420 mucosa. Except for 30 min incubation with PGM dispersion, which increased nicotine permeability,

no other treatments significantly affected the permeability of nicotine compared to the permeability across non-incubated tissue. This could be explained by lack of interactions between the tissue and the applied mucin. The extent of interaction could be decreased by damage of the mucosal surface from handling, such as freezing, thawing and slicing of the tissue. Bio-incompatibility between the tissue and the applied mucin could also affect the degree of interaction. Another possible explanation for the lack of effect on nicotine permeability could be that the epithelium constitutes a much larger barrier to nicotine, hence a possible effect from the mucin dispersions become negligible in comparison.

3.2.4 Total drug recovery

After the permeability studies the total recovery of the four model drugs was determined. The results are presented in Table 4. For nicotine, mannitol and caffeine the recoveries were acceptable, whereas, the recovery of propranolol was lower.

The relatively poor recovery for propranolol is likely due to adsorption to the Ussing chamber walls and cell inserts. It has previously been shown that diazepam, a lipophilic small molecule, adsorbed to Ussing chamber walls during transport studies [44]. In the cell studies the recovery was initially measured without adding propranolol content in the filter-grown cells, as these were used in viability testing; however, the recovery of propranolol was only 56.5% (Table 4). Therefore, an additional experiment with propranolol was performed, where the propranolol content of the filter and cells was added to the recovery resulting in a propranolol recovery of 91.4% (Table 4). It is likely that propranolol, due to its lipophilic nature, was mostly located in the lipophilic cell layers. Other studies have also shown that approximately 30% of propranolol was located in filter-grown TR146 cells after a permeability study [24].

4 Conclusions

444 The pH of the HFDS dispersion and its shear thinning properties is more similar to native oral mucus
445 than PGM, thus HFDS is more suitable for mimicking the mucus layer in the oral cavity.

446 A new one compartment device has been applied to study drug permeability across an isolated mucin
447 dispersion or saliva buffer. Four model drugs showed decreased permeability across mucin
448 dispersions and a greater effect was seen with HFDS than with PGM. The effect on drug permeation
449 could be caused by differences in viscosity between the mucin dispersions and the saliva buffer or by
450 interactions between drug molecules and mucin.

451 TR146 cells were subjected to mucin dispersions 24 h prior to a permeability study. This model may
452 become a promising *in vitro* method to study drug permeation across a mucus layer in conjunction
453 with a multi-layered epithelium. The integrity and viability of the TR146 cells were maintained during
454 24 h incubation and a subsequent permeability study. Permeability of propranolol and caffeine was
455 decreased by the presence of mucin, however, this was not shown for nicotine and mannitol. The
456 HFDS attached to the TR146 cell surface to a higher extent than PGM, however, the strength and
457 mechanism behind the attachment needs further studies.

458 Incubation of porcine buccal mucosa with mucin dispersions for 24 h caused compromised integrity
459 of the tissue. Mannitol permeability across non-incubated tissue and tissue incubated for 30 min was
460 too low to be quantified. Tissue incubation with mucin dispersions did not decrease nicotine
461 permeability, indicating that the epithelium constitute the main barrier for nicotine diffusion across
462 porcine buccal mucosa. Further studies are needed to determine whether it is possible to reintroduce
463 mucin molecules on the tissue surface.

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